Historic, archived document

Do not assume content reflects current scientific knowledge, policies, or practices.

LIBRARY
CURRENT SERIAL RECORD
★ JAN 31 1946 ★
U.S. DEPARTMENT OF AGRICULTURE

January 1946 AIC-106

PRODUCTION, CONCENTRATION, PROPERTIES, AND ASSAY OF THE ANTIBIOTIC, SUBTILIN

Western Regional Research Laboratory, Albany, California
Bureau of Agricultural and Industrial Chemistry
Agricultural Research Administration
U. S. Department of Agriculture

Subtilin is an antibiotic produced by some strains of the bacterium Bacillus subtilis under the proper culturing conditions. This antibiotic is being investigated at the Western Regional Research Laboratory in connection with studies on the utilization of waste vegetable materials as bacterial culture media. Production of subtilin has been carried out by the use of culture media prepared from waste asparagus butts.

Subtilin has been purified to a point where it is approximately 100 times as active as the original pellicle (dry basis). Experience has shown the product to possess a relatively low order of toxicity and it is believed suitable for experimentation to determine its therapeutic usefulness. The antibiotic activity and therapeutic usefulness of the concentrate described below is being studied at the University of California. This report deals with essential facts regarding production, concentration, and assay of the active material as carried out in this Laboratory.

Production of the Pellicle

The subtilin-producing bacillus is grown on asparagus-butt-juice concentrate diluted with tap water to approximately 8 percent total solids. Best results have been obtained with asparagus concentrates prepared by the digestion process (3).[1] The concentrate should be stored under refrigeration; after several months at room temperature, darkening and formation of inhibitors of microbial growth have been observed.

Aliquots (500 ml.) of asparagus medium are dispensed into Fernbach flasks, which are then steamed at atmospheric pressure for 20 minutes. Steaming achieves practical sterilization of the medium. The spore-bearing rods which survive do not interfere during the short incubation period used. After the flasks are cool the pH is adjusted to 6.5 with sterile 3 N NaOH; approximately 2.5 ml. is required per flask. The pH is adjusted after rather than before sterilization because sterilization is more effective at the unadjusted pH (approximately 5).

Subtilin is produced by the strain of Bacillus subtilis designated in the stock culture collection of the Northern Regional Research Laboratory as B-543 (1, 2). This is the only one of several strains of B. subtilis tested in the Western Regional Laboratory that produces subtilin. Stock cultures of the organism are carried on agar slants of "Medium II" of Schmidt and Moyer (5). After continued subculture on this medium the organisms grow as a non-adherent film, whereas on nutrient agar the film adheres strongly. The cultures are incubated overnight at 35° C. and stored in a refrigerator between transfers.

---

[1] Numbers in parentheses refer to literature listed on page 7.

Inoculum is prepared by washing the growth from about 25 sq.cm. of freshly incubated slants into 500 ml. of asparagus medium. This culture is incubated at 37° C. for 24 hours, or for at least 4 hours after the pellicle becomes visible. The pellicle is then broken up sufficiently for pipetting by swirling with sterile glass beads. Production flasks are inoculated with 1 percent by volume of the inoculum culture.

Production cultures are incubated at 37° C. for 28 hours, or until a heavy pellicle is formed. The pellicle normally is tan in color but occasionally is green or has a greenish cast. The green color is not due to microbial contamination. The pellicles are harvested by straining the cultures through cheesecloth. The culture liquor is discarded, since it contains only a minor fraction of the total antibacterial activity.

Concentration of Subtilin

The harvested pellicle, which is composed of B. subtilis cells plus asparagus juice medium, contains about 10 percent solids on the volume basis. About 100 ml. of the cells are obtained from each liter of culture.

The moist cells are extracted by stirring with 95-percent alcohol. Sufficient alcohol is added to the mixture to bring the concentration of alcohol up to 60-70 percent. At this concentration the cellular debris is precipitated and all of the colloidal material is flocculated, leaving a clear supernatant liquor.

After standing overnight in a cold room (4° C.) about two-thirds of the supernatant liquor may be siphoned off. The cells are then removed from the remaining extract by suitable filtration. A basket centrifuge with canvas filter has been found useful.

Sufficient glacial acetic acid is added to the clear alcohol extract to make a 1 percent solution. The extract is concentrated in vacuo at a temperature of 30° C. or below. After about 80 percent of the solvent has been removed, the concentrated liquor is decanted and the precipitated solids allowed to settle in a cold room at 4° C. The precipitate which settles out is collected by centrifugation. Sufficient sodium chloride is added to the supernatant liquor to bring the concentration to about 15 percent. This precipitates more material from solution, which is recovered by centrifugation. The precipitate is washed by suspension and centrifugation 3 to 4 times with 20 percent ammonium chloride solution. Ammonium chloride is used to prevent the re-dissolving of the active material, and to wash the precipitate free from sodium chloride. The ammonium chloride-washed precipitate is filtered on a Büchner funnel with hard filter paper. The major portion of the ammonium chloride solution is pressed from the precipitate by a thin rubber membrane placed over the funnel and allowed to be sucked down tight against the filter cake.

The distilled water-washed precipitate and the ammonium chloride-washed precipitate are combined, frozen, and dried from the frozen state. The dried precipitates are ground carefully to a fine powder. This material has been designated as the *salt cake*.

The dried salt cake is weighed and sampled for assay and is then thoroughly extracted by suspension and centrifugation with 95-percent alcohol. Subtilin is quite insoluble in 95-percent alcohol and is collected in the salt-free precipitate. About 500 ml. of alcohol is used in 5 portions of 100 ml. each for the extractions of the cake obtained from 100 liters of original culture.

The extracted cake is dried by vacuum desiccation as before. The dried material is ground to a fine powder, weighed and sampled for assay. This preparation has been designated as the *alcohol-extracted cake*.

Table 1 gives the results obtained from a typical isolation experiment. The values given in the column designated "total mg. subtilin" are obtained from the microbiological assay. They represent the equivalent weight of a standard reference subtilin sample designated as L-1242, which is the more active subtilin preparations we have obtained, as determined by its activity against *Staphylococcus aureus*.

Table 1. Concentration of Subtilin.

Fraction	Volume (liters)	Dry weight (gm.)	Total subtilin (mg.)
Cell medium	96.5	--	--
Moist pellicle	9.2	750	--
Alcohol extract	17.4	322	12,400
Salt cake	--	33.8	12,400
Alcohol-extracted salt cake	--	14.6	12,000

The alcohol-extracted cake is then subjected to an acetic acid fractionation, in which a dark gummy water-insoluble inactive material is separated from the water-soluble fraction. This is done as follows: A 20-25-percent solution of the alcohol-extracted cake is made in glacial acetic acid. This solution is chilled and sufficient ice water is added with stirring to make a final concentration of 25 percent acetic acid by volume. A precipitate is immediately thrown out of solution and is removed by centrifugation. The gummy black precipitate is redissolved in glacial acetic acid as before. The solution is chilled and again treated with ice water sufficient to make a 25-percent solution of acetic acid. The precipitate is removed and re-extracted once more in the same manner. The material soluble in 25-percent acetic acid is collected by drying from the frozen state.

Approximately 60 percent of the alcohol-extracted salt cake is extracted by the acetic acid. The activity of the extracted material is higher than that of the starting material but represents only about 70 to 80 percent of the total activity. The importance of this step lies primarily in the fact that the product obtained is a readily soluble powder, whereas the material removed by precipitation is a dark brown gummy substance which is very insoluble in aqueous solutions and which previous to its removal held the active material very tenaciously. An appreciable amount of subtilin remains in this gummy material after precipitation. Methods for the recovery of this adsorbed material have not been satisfactorily worked out at present.

Subtilin is a low-molecular-weight substance which dialyzes through Cellophane membranes. The final purification step at present consists of dissolving the dried acetic acid-soluble material in distilled water (5 to 10 percent solution) and dialyzing against a 1-percent acetic acid solution with stirring at 4° C. The outside solution is removed and replaced with fresh solvent at 48-hour intervals and the active material is recovered by lyophilization. An amount of dark brown non-dialyzable material remains in the bag. The final preparation is a light-brown amorphous powder, which is readily soluble in water below pH 7.0. It consists of low-molecular-weight substances, insoluble in 95-percent alcohol, but readily soluble in 70-percent alcohol. The substances present are further characterized by being precipitated at low salt concentrations and by being soluble to an appreciable extent only in acid solution. Subtilin is therefore probably a basic substance giving water-soluble salts.

Solubility of Subtilin

Approximate solubility in aqueous solutions--

Water, pH 2.0 to 6.0	at least 10 percent
Water, pH 7.0	0.5 percent
Water, pH 8.5	0.2 percent
Water, pH 4.5, plus 1 percent salt	0.2 to 0.5 percent
Water, pH 4.5, plus 20 percent salt	0.001 percent

Approximate solubility in alcohol solutions--

Alcohol plus water, 0-80 percent alcohol	10 percent
95-percent alcohol	0.3 percent
Absolute alcohol	0.3 percent

Approximate solubility in acetic acid--

Acetic acid plus water, 0-100-percent acetic	10 percent

Solubility in other solvents--

Acetone	Insoluble
Ether	Insoluble
Methanol (acid pH)	Moderately soluble
Petroleum ether	Insoluble
Chloroform	Insoluble
Amyl acetate	Insoluble

Stability of Subtilin

Subtilin is stable in dried form. Samples stored at room temperature in the dark have retained their activity for months. Light has a destructive action on subtilin, particularly when it is in solution. In direct sunlight the activity of aqueous or alcoholic solutions at pH 4.0 is lost rather rapidly, whereas in the dark such solutions are stable for relatively long periods. A dilute solution of subtilin (40 micrograms per milliliter) at pH 2.5 in the refrigerator retained 90 percent of its activity for three months. Before subtilin solutions are dried they should be acidified with acetic acid (below pH 5.0); hydrochloric acid must not be used, since complete destruction of activity takes place on drying in its presence. Subtilin preparations are uniformly stored in the refrigerator as the dried powder obtained from drying solutions acidified with acetic acid from the frozen state.

Table 2 gives the relative stability of subtilin at 25° C. with regard to pH. It is stable in acid solution but unstable at alkaline reaction. Table 3 gives additional information on the stability within the pH range of 6.8 to 9.0 at 37° C.

Table 2. Stability of Subtilin in Solutions at Varied pH (25° C.) (Time period, 24 hours)

pH of solution	Activity retained
	percent
4.5	98
5.5	98
6.0	98
7.0	94
7.5	86
8.0	80
8.5	84
9.0	65
9.6	30
10.0	13
11.2	4.5

Table 3. Stability of Subtilin in Solutions at Varied pH (37° C.)

pH of solution	Activity retained 1 day	Activity retained 6 days
	percent	percent
6.8	65	43
7.3	64	32
7.7	58	21
8.2	46	12
8.6	27	6
9.0	15	5

Proteolytic enzymes have been found to inactivate subtilin. This was found to be true of pepsin, trypsin, and crude pancreatic enzymes.

Microbiological Assay of Subtilin

The retention and concentration of subtilin during purification are followed by means of a 4-hour turbidimetric growth-inhibition assay with Staph. aureus (Merck's strain H). The method is a modification of that described by McMahan (4) for the assay of penicillin. "Medium II" is used instead of that described by McMahan because it permits a higher level of growth and because it is less colored and more highly buffered. It is made up 50 percent more concentrated than that described by Schmidt and Moyer (5) so that the normal strength is obtained on addition of 10 ml. of inoculated medium to culture tubes containing 5 ml. of aqueous solution of standard or unknown.

The subtilin standard and unknown samples are diluted to the desired concentration with distilled water adjusted to pH 2.5 with hydrochloric acid, because the stability of the dilute solutions required is enhanced. The acid does not affect the growth of the organism in the buffered basal medium. The samples are distributed into 18 x 150 mm. Pyrex test tubes with an automatic pipetting machine (Baltimore Biological Laboratory) set to deliver 1-ml. aliquots. For each sample, tubes are filled, in triplicate, with 1, 2, 3, 4, and 5 1-ml. aliquots. After this is accomplished with all samples, the volume is made to 5 ml. by distributing the appropriate number of 1-ml. aliquots of pH 2.5 hydrochloric acid diluent.

We have obtained less erratic results by the use of unsterile culture tubes than by the use of tubes which had been exposed to the gases of an oven.

After the samples have been distributed, a 10-ml. aliquot of inoculated basal medium is added to each tube with an automatic pipetting machine and, with a minimum of delay, the entire set of tubes is submerged in a well-stirred water bath at 37° C. After four hours of incubation the tubes are removed, covered with folded towels, and autoclaved. Photometric turbidity readings are made on the following day because the cultures undergo an appreciable change in turbidity within the first hour or two after autoclaving.

Staph. aureus is carried on Medium II agar slants. On the afternoon before an assay, a loop scraping of Staph. aureus is transferred to 150 ml. of Medium II and incubated at 37° C. On the following day, 24 ml. of this culture (now approximately 18 hours old) is added per liter of Medium II (150 percent strength), which has been refrigerated. After mixing, this medium is added to the assay samples.

The biological response to a given amount of subtilin varies with the length of time during which the subtilin is in contact with the inoculated culture medium before the tubes are placed in the bath at 37° C. This variation is minimized but not eliminated by the use of refrigerated medium. For this reason it is necessary to repeat the standard every 4 or 6 samples, and to follow a systematic order in filling and handling the assay tubes. After the turbidities have been measured, the shift in the standard is plotted against position in the assay setup and assay values for unknown samples are corrected accordingly. The shift between the standards at the ends of a setup with 18 samples usually varies between 10 and 20 percent.

The most active preparations obtained to date have given the range of zero to complete inhibition between approximately 0.5 and 4 micrograms per 15 ml. of culture. Different preparations sometimes give dosage-response curves with markedly different slopes, demonstrating a multiplicity of components which affect the growth of _Staph. aureus_ under our conditions. Obviously, assay values for one type of material calculated against a standard curve obtained with another type of material will show trends with size of aliquot; such assay values are of limited significance. With samples of the same type that do not contain interfering substances it is possible to recognize differences in potency as low as 5 percent. The method as routinely used has given a reproducibility of approximately 10 percent.

Of the fractions obtained in the purification of subtilin, the soluble fraction obtained by extraction of the "salt cake" (Table 1) with 95-percent ethanol gave the worst interference with the assay of subtilin. The direct assay of subtilin in this fraction was not possible, and the addition of small amounts of this fraction influenced the assay of other fractions.

The sigmoidal dosage-response curves become almost linear when percentage response is plotted against dosage on arithmetic-probability paper (Keuffel and Esser Co., No. 358-23).

Literature Cited

1. Humfeld, H. and Feustel, I. C. Utilization of asparagus juice in microbiological culture media. Proc. Soc. Expt. Biol. and Med. 54(2):232-235. 1943.

2. Jansen, E. F. and Hirschmann, D. F. Subtilin--an antibacterial product of _Bacillus subtilis_. Arch. Biochem. 4(3):297-309. 1944.

3. Lewis, J. C., Dimick, K. P., and Feustel, I. C. Production of tyrothricin in cultures of Bacillus brevis. Indus. and Engin. Chem., 37(10):996-1004. 1945.

4. McMahan, J. R. An improved short time turbidimetric assay for penicillin. Jour. Biol. Chem. 153(1):249-258. 1944.

5. Schmidt, W. H. and Moyer, A. J. Penicillin. I. Methods of assay. Jour. Bact. 47(2):199-208. 1944.

LIBRARY
CURRENT SERIAL RECORD
FEB 5 1946 ★
U. S. DEPARTMENT OF AGRICULTURE